

Detection of Adenoviruses and Enteroviruses in Polluted Waters by Nested PCR Amplification

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A procedure has been developed for the rapid detection of enteroviruses and adenoviruses in environmental samples. Several systems for virus concentration and extraction of nucleic acid were tested by adding adenovirus type 2 and poliovirus type 1 to different sewage samples. The most promising method for virus recovery involved the concentration of viruses by centrifugation and elution of the virus pellets by treatment with 0.25 N glycine buffer, pH 9.5. Nucleic acid extraction by adsorption of RNA and DNA to silica particles was the most efficient. One aliquot of the extracted nucleic acids was used for a nested two-step PCR, with specific primers for all adenoviruses; and another aliquot was used to synthesize cDNA for a nested two-step PCR with specific primers for further detection of seeded polioviruses or all enteroviruses in the river water and sewage samples. The specificity and sensitivity were evaluated, and 24 different enterovirus strains and the 47 human adenovirus serotypes were recognized by the primers used. The sensitivity was estimated to be between 1 and 10 virus particles for each of the species tested. Twenty-five samples of sewage and polluted river water were analyzed and showed a much higher number of positive isolates by nested PCR than by tissue culture analysis. The PCR-based detection of enteroviruses and adenoviruses shows good results as an indicator of possible viral contamination in environmental wastewater.

A very large number of different viruses are excreted in human feces and urine and have been found in sewage and polluted waters (22, 28, 38). Enteric viruses include recognized viral pathogens involved in important diseases, such as Norwalk virus, rotavirus, hepatitis A virus, adenovirus, and enterovirus infections (7). The most commonly studied virus groups in polluted waters are the members of the *Picornaviridae* family and, more specifically, the genus *Enterovirus*, which includes poliovirus, coxsackievirus A and B, echovirus, and other enteroviruses. Enteroviral infection can lead to a broad spectrum of manifestations, ranging from asymptomatic infection to serious disease and fatality (29, 30). The presence of enteroviruses in the environment is a public health hazard (33) even when very few viral particles are present (36).

The detection of viral pathogens by cell culture is very complex, and not all groups of viruses can be isolated on regular cell lines. PCR is an *in vitro* method for primer-directed enzymatic amplification of specific target DNA sequences (34, 35) which provides very sensitive, specific, and rapid detection of viruses in a variety of environmental samples (1, 26). However, it requires the design of specific primers, and the development of a method for recovery, concentration of viral particles followed by nucleic acid extraction, and purification from complex environmental samples, which should also eliminate potential inhibitors of the reverse transcriptase (RT) reaction and PCR amplification. Nested PCR amplification was applied in this study to ensure the specificity of detection, eliminate any false-positive results, and increase the amplification signal, providing the method with the highest sensitivity,

which allows us to detect a small number of viral contaminants in environmental samples.

Enteroviruses and adenoviruses were chosen for this study for the following reasons. The enteroviruses have been used as a parameter for evaluating the viral pollution of the environment, since most of them can be isolated and quantified as PFU in cell culture (32). Some authors have suggested the use of poliovirus for viral monitoring because of its prevalence as a component of the human vaccine, but poliovirus is not always detected in wastewater. The U.S. Environmental Protection Agency describes the enteric virus group itself as the most meaningful, reliable and effective virus index for environmental monitoring (25). Enteroviruses are the only group contemplated in the guidelines of the European Communities for the aquatic environment. Adenoviruses are the only human enteric viruses to contain DNA and are important human pathogens. Many adenovirus serotypes are difficult to culture in regular cell lines. For this reason and because adenoviruses are slow growing, their presence in polluted water and their role as originators of gastroenteritis have probably been underestimated (20, 23, 27). Subgenus F of adenovirus (serotypes 40 and 41) (15) is called "fastidious" because of the difficulty of its isolation, and both serotypes are almost as important as rotavirus as etiological agents of infantile gastroenteritis (7, 11, 39, 40).

We propose here a complete procedure for virus concentration from sewage and from river water, nucleic acid extraction, and the detection of the specific viruses by amplification of DNA or cDNA with the appropriate primers. Two groups of totally different viruses were selected for this study in order to develop a method that could be used for the detection of a wide variety of viral contaminants. The enteroviruses and adenoviruses detected by PCR were evaluated as indicators of possible viral contamination of environmental wastewater samples.

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TABLE 1. Origins and characteristics of adenovirus prototype and prototype-like strains used in the PCR amplification

Serotype	Subgenus	Strain	Description	Original source of isolation
Ad1	C	YoR/Chile	Genome type 1p	Adenoid
Ad2	C	Ad6	Prototype	Adenoid
Ad3	B ₁	GB	Prototype	Nasal washing
Ad4	E	R167	Prototype	Throat washing
Ad5	C	Ad75	Prototype	Adenoid
Ad6	C	Ton99	Prototype	Tonsils
Ad7	B ₁	Gomen	Prototype	Throat washing
Ad8	D	Trim	Prototype	Eye swab
Ad9	D	Hicks	Prototype	Stool
Ad10	D	J.J.	Prototype	Eye swab
Ad11	B ₂	Slobitski	Prototype	Stool
Ad12	A	F-3072/86	Prototype-like	Stool
Ad13	D	A.A.	Prototype	Stool
Ad14	B ₂	DeWitt	Prototype	Throat swab
Ad15	D	Ch38	Prototype	Eye swab
Ad16	B ₁	Wigand	Prototype-like	Eye swab
Ad17	D	Ch22	Prototype	Eye swab
Ad18	A	D.C.	Prototype	Anal swab
Ad19	D	587	Prototype	Conjunctiva
Ad20	D	931	Prototype	Conjunctiva
Ad21	B ₁	SBL	Prototype-like	Conjunctiva
Ad22	D	2711	Prototype	Conjunctiva
Ad23	D	SBL	Prototype-like	Conjunctiva
Ad24	D	3153	Prototype	Conjunctiva
Ad25	D	BP-1	Prototype	Anal swab
Ad26	D	BP-2	Prototype	Anal swab
Ad27	D	BP-4	Prototype	Anal swab
Ad28	D	BP-5	Prototype	Anal swab
Ad29	D	BP-6	Prototype	Anal swab
Ad30	D	BP-7	Prototype	Anal swab
Ad31	A	1315/63	Prototype	Stool
Ad32	D	H.H.	Prototype	Anal swab
Ad33	D	D.J.	Prototype	Anal swab
Ad34	B ₂	259	Prototype-like	Urine
Ad35	B ₂	12221/80	Prototype-like	Lung and kidney
Ad36	D	275	Prototype	Stool
Ad37	D	GW	Prototype	Eye
Ad38	D	70/17368	Prototype-like	Stool
Ad39	D	81/13027	Prototype-like	Stool
Ad40	F	Hovi X	Prototype	Stool
Ad41	F	Tak	Prototype	Stool
Ad42	D	Paris 54	Prototype	Stool
Ad43	D	1309	Prototype	Stool and urine
Ad44	D	1584	Prototype	Stool
Ad45	D	1590	Prototype	Stool
Ad46	D	1594	Prototype	Stool, bronchial brush
Ad47	D	1601	Prototype	Stool

MATERIALS AND METHODS

Viruses and cells. Adenovirus type 2 (Ad2) (prototype) and Ad12 (prototype-like) were grown on HEp-2 cells and poliovirus type 1 (LSc strain) was propagated in Buffalo green monkey kidney (BGM) cells growing in MEM Thermo-pow (JRH Biosciences) containing 5% fetal bovine serum.

The 24 different enterovirus strains used in specificity experiments were all wild-type strains which had been isolated from patients and typed according to neutralization tests with specific sera provided by the National Institute of Allergy and Infectious Diseases, and they were grown on LLC-MK₂ cells, using Dulbecco's modification of Eagle's minimal essential medium (MEM) (GIBCO) containing 5% fetal bovine serum. The specificities of the external and internal adenovirus primers were checked by using one prototype strain of each one of the 47 human adenovirus serotypes (Table 1).

Infectious enteroviruses from the samples were grown and assayed as PFU in BGM cell monolayers, the standard cell line used to assay environmental samples for enterovirus (5). Briefly, BGM cells were grown to confluent monolayers in 75-cm² plastic flasks. Before exposure to the sample, the growth medium was poured off and 1 ml of sample was inoculated into each 75-cm² plastic flask. The flasks were incubated at 37°C for 60 min and were gently rotated every 15 min to allow virus adsorption to the cells. The cells were overlaid with MEM Thermo-pow (JRH Biosciences) with 2% fetal bovine serum and 1% agar. After 5 to 6 days of incubation at 37°C in 5% CO₂ in air, the cells were stained with neutral red in MEM and plaques were counted.

Adenovirus detection in cell culture was carried out by infecting flasks of 75 cm² containing HEp-2 cells with 250 µl of the viral particles concentrated from the sewage samples. One hour before the infection, the culture medium was replaced by

inoculum medium containing MEM Thermo-pow (JRH Biosciences) supplemented with penicillin (400 U/ml), streptomycin (400 µg/ml), and guanidine hydrochloride (100 µg/ml) to inhibit enterovirus growth (19). Infection was allowed to proceed for 1 h at 37°C. Then, 25 ml of inoculum medium was added. The medium was changed after 24 h and every 2 days thereafter, until cytopathic effects were observed, or until the cells were too old, in which case a second flask was infected with 100 µl of the contents of the first flask after freezing and thawing of the cells five times.

Environmental samples. Twenty independent untreated domestic sewage samples were studied to compare the adenovirus results by nested PCR and by tissue culture plus one-step PCR with external primers. After the treatment for the recovery and concentration of viral particles, these samples were resuspended in 0.5 ml of phosphate-buffered saline (PBS) and kept at -80°C, and 50 µl was later used for DNA extraction; 250-µl aliquots were used to infect HEp-2 cells as described above.

Sixteen untreated domestic sewage samples and nine Llobregat River water samples were analyzed for enterovirus by PFU count and nested PCR and for adenovirus by nested PCR. For enterovirus quantification by PFU count, 10 or 20 ml of the sample, if available, was used to infect BGM cells after chloroform decontamination of the sample.

Concentration of viral particles. River water samples of 50 liters were collected in the Llobregat River, and viral particles were concentrated by the adsorption-elution glass powder method of Schwartzbrod and Lucena (37). The viruses were eluted with 0.25 N glycine buffer, pH 9.5, containing 3% beef extract, and the 60-ml volume of viral suspension recovered was treated in the same way as the sewage samples.

Preparation of viral particles for nucleic acid extraction. This procedure is applied to reduce the volume of both very polluted water and viral concentrates from water samples with low pollution levels. Previously described techniques for the recovery of adenovirus particles from sewage (16) were applied to enterovirus detection. Sewage samples were treated in four ways after adding serotypes Ad2 and Ad12 (prototypes) and poliovirus type 1, in order to select the most efficient method for virus recovery and PCR detection. In each experiment, 10⁷ virus particles were added to 150 ml of the sample and after 30 min of continuous mixing, the aliquots were separated for the different treatments.

In the first treatment assayed, 30 ml of the sewage sample was filtered through a low-protein-binding filter (Sterivex-GV; Millipore). The filter was previously treated with 3 ml of beef extract (3%, pH 9.5). The viruses retained were eluted by passing 2 ml of glycine buffer (0.25 N, pH 9.5) through the membrane several times in both directions for 10 min. The resulting 32-ml volume was centrifuged at 48,400 × *g* for 3 h 45 min at 4°C and the virus pellets were resuspended in 0.5 ml of PBS and kept at -80°C, as were the viruses recovered by the following treatments.

For the second treatment assayed, the 30-ml aliquots were centrifuged at 48,400 × *g* for 3 h 45 min at 4°C in order for all the viruses to form a pellet with the suspended material. The pellet was resuspended in 0.5 ml of PBS.

In the third treatment, suspended solids were first separated by centrifuging 30 ml of the sample at 12,100 × *g* for 15 min. The viruses retained in the pellet were eluted by mixing it with 5 ml of 0.25 N glycine buffer, pH 9.5, on ice for 30 min, and then 25 ml of PBS was added and the solids were separated by centrifugation at 12,100 × *g* for 15 min. Both supernatants were centrifuged at 48,400 × *g* for 3 h 45 min at 4°C in order

to form pellets of the virus particles, which were finally resuspended in 0.5 ml of PBS, as described for the other samples.

The fourth treatment started with the centrifugation of 30 ml of sample to form pellets of all the viral particles with any suspended material (48,400 × *g* for 3 h 45 min at 4°C). The viruses retained in the pellet were eluted by mixing it with 5 ml of 0.25 N glycine buffer, pH 9.5, on ice for 30 min, and then 25 ml of PBS was added and the solids were separated by centrifugation at 12,000 × *g* for 15 min. The virus suspensions obtained were ultracentrifuged at 171,360 × *g* for 1 h at 4°C, to form pellets of the viral particles, which were resuspended in 0.5 ml of PBS and kept at -80°C until processing for nucleic acid extraction and PCR detection.

Untreated sewage collected from the suburban network of Barcelona, Spain, and concentrated viral particles from the river water samples were treated according to the fourth method described. Two aliquots of 40 ml were thus finally resuspended in 0.1 ml of PBS each.

Nucleic acid extraction. Three methods for nucleic acid extraction were compared. Sewage water was supplemented with poliovirus type 1 or Ad2 (prototype).

The first method was that described by Chomczynski and Sacchi (10), which uses guanidinium thiocyanate (GuSCN)-phenol-chloroform for nucleic acid extraction and ethanol precipitation of the nucleic acids. The second method was that described by Boom et al. (8), which uses GuSCN and adsorption to silica particles. The third method was that described by Yamada et al. (42), which uses a treatment with GuSCN and nucleic acid adsorption to glass powder.

The results were compared by quantification of the recovered nucleic acids by measuring *A*₂₆₀ and by comparing the intensities of the DNA bands in agarose after PCR amplification.

The method of Boom et al., with minor modifications, was applied to the field samples, and the procedure was started with 50 µl of viral suspension. This volume of sample was added to a mixture of 40 µl of the silica particle suspension and 900 µl of lysis buffer (120 g of GuSCN in 100 ml of 0.1 M Tris-HCl, pH 6.4, with 22 ml of 0.2 M EDTA adjusted with NaOH to pH 8.0 and 2.6 g of Triton X-100 added), left for 10 min at room temperature, and washed twice in 1 ml of washing buffer (120 g of GuSCN in 100 ml of 0.1 M Tris-HCl, pH 6.4), twice more with ethanol 70%, and once with acetone. The pellet obtained after the complete evaporation of acetone was resuspended with 50 µl of 10 mM Tris-HCl, pH 7.6-0.1 mM EDTA, pH 8.0-1 mM dithiothreitol with RNasin (an RNase inhibitor) (5-U/µl final concentration) in order to allow nucleic acid elution from silica particles. The resulting supernatant was used in cDNA synthesis for enteroviruses and PCR amplification for adenoviruses.

Specific primers. The specific primers for detection of human adenovirus were selected from the DNA sequence of the open reading frame of hexon genes of Ad2, Ad40, and Ad41 and have been described in previous reports (3, 4). The specific primers for enterovirus detection were selected from the 5' nontranslated region of the enterovirus genome, aligned with previously published sequences (6, 9, 17, 21, 24), and evaluated against the sequences of the EMBL data bank by the FastA program of the Genetics Computer Group package (12) (Table 2). The specificity of the primers was evaluated against 24 enterovirus strains and the 47 human adenovirus serotypes as indicated above.

Sensitivity of the enterovirus primers. The sensitivities of the two sets of primers for the detection of enterovirus by PCR amplification were checked by a limiting dilution experiment. Serial 10-fold dilutions of the supernatants of coxsackievirus B2-, echovirus 11-, and poliovirus type 1-infected LLC-MK₂

TABLE 2. Oligonucleotide primers used in this study for PCR amplification of adenoviruses and enteroviruses

Virus type (region) ^a	Position	Amplification reaction	Primer	Sequence	<i>T_m</i> (°C) ^b	Product size (bp)
Ad2 (hexon)						
Ad40 (hexon)	18858–18883 ^c	First	hexAA1885	5'-GCCGCAGTGGTCTTACATGCACATC-3'	78	300
Ad41 (hexon)	19136–19158 ^c	First	hexAA1913	5'-CAGCACGCCGCGGATGTCAAAGT-3'	74	
Ad2 (hexon)	18937–18960 ^c	Nested	nehexAA1893	5'-GCCACCGAGACGTACTTCAGCCTG-3'	78	142
Ad2 (hexon)	19051–19079 ^c	Nested	nehexAA1905	5'-TTGTACGAGTACGCGGTATCCTCGCGGTC-3'	92	
Polio 1 (5' NTR)						
CV B4 (5' NTR)	64–83 ^d	First	Ent1 ^e	5'-CGGTACCTTTGTACGCCTGT-3'	62	534
Polio 1 (5' NTR)	578–597 ^d	First	Ent2	5'-ATTGTACCATAAGCAGCCA-3'	58	
Polio 1 (5' NTR)	430–450 ^d	Nested	neEnt1	5'-TCCGGGCCCTGAATGCGGCTA-3'	70	138
CV B4 (5' NTR)	547–567 ^d	Nested	neEnt2	5'-GAAACACGGACACCCAAAGTA-3'	62	

^a Ad, adenovirus; polio, poliovirus; CV, coxsackievirus; NTR, nontranslated region.

^b Melting point temperatures (*T_m*s) were calculated by the equation $T_m = 4 \times (\text{number of GC base pairs}) + 2 \times (\text{number of AT base pairs})$ (41).

^c The sequence positions refer to the Ad2 hexon region (2).

^d The sequence positions refer to the coxsackievirus B4 5' NTR (24).

^e Modified from that of Gow et al. (17).

cells (rhesus monkey kidney cells) were used to infect LLC-MK₂ monolayers in order to determine the virus titer by the plaque assay technique (31). The amount of virus corresponding to a single PFU was further serially diluted, and total RNA was extracted from aliquots containing from 10⁻¹ to 10⁻⁴ PFU. This was reverse transcribed and amplified by the two-step PCR described below.

cDNA synthesis of enterovirus RNA. The reaction mixture for reverse transcription had a total volume of 10 µl and contained 5 µl of the nucleic acids extracted plus 1.5 mM MgCl₂, 1× PCR amplification buffer (10× buffer contains 50 mM KCl, 10 mM Tris-HCl [pH 9.0, at 25°C], 0.01% [wt/vol] gelatin, and 0.1% Triton X-100), deoxynucleoside triphosphates at 200 µM each, 200 U of Moloney murine leukemia virus RT (Promega), and 2.5 µM external primer Ent2 for enterovirus (Table 2). The reaction mixture was incubated at 95°C for 5 min before the addition of the enzyme and RNasin. The temperature cycle was set for 30 min at 42°C and 5 min at 95°C.

To reduce the probability of sample contamination by amplified enteroviral DNA molecules, separate areas were used for reagents and amplified samples and, after the enteroviral cDNA synthesis reaction had finished, 2 U of the restriction enzyme *AluI* (Boehringer Mannheim) was added to the mixture, which was incubated for 1 h at 37°C. The restriction enzyme was active in the PCR buffer and was deactivated at 95°C for 5 min. The rest of the mixture for the PCR amplification was then added to the same tube. *AluI* is a restriction enzyme that cuts double-stranded DNA with a recognition site of only 4 bases and, for instance, cuts the first amplicon of poliovirus type 1 into at least three different fragments (data not shown).

Enzymatic amplification of DNA and cDNA. For a typical one-step reaction, 10 µl of extracted viral DNA (corresponding to 4 ml of the sewage sample and 4 liters of the river water sample) was used for adenoviruses and 10 µl of the cDNA solution (corresponding to 2 ml of the sewage sample and 2 liters of the river water sample) was used for enteroviruses. Amplification was carried out in a 50-µl reaction mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25°C), 1.5 mM MgCl₂, 0.01% gelatin (wt/vol), 0.1% Triton X-100, deoxynucleoside triphosphates (i.e., dATP, dCTP, dGTP, and

dTTP or dUTP for the enterovirus or adenovirus reaction, respectively) at 200 µM each, each adenovirus primer at 0.08 µM or each enterovirus primer at 0.5 µM, and 2 U of thermostable *Taq* DNA polymerase (Promega). The primer concentration was selected after comparison of the amplification results from tests of four different concentrations of primers (data not shown). Enterovirus detection required high concentrations of Ent2 in the cDNA synthesis step and also consequently Ent1 in the first 30 cycles of amplification. The samples were overlaid with 75 µl of mineral oil to prevent evaporation. Thermal cycling of the amplification mixture was performed in a programmable heat block (Coy Laboratory Products, Inc., Ann Arbor, Mich.). In all PCR assays, the first cycle of denaturation was carried out for 4 min at 94°C. The conditions for amplification consisted of denaturing at 92°C for 90 s, annealing at 55°C for 90 s, and extension at 72°C for 120 s.

The reaction mixture for adenovirus PCR amplification contained dUTP instead of dTTP and was treated with 1 U of uracil DNA glycosylase for 1 h at 37°C (19) in order to eliminate contamination with previously amplified DNA.

The external primers were used in the first 30 cycles of amplification, and 1 µl (1/50 dilution) was further added to a new batch of 50 µl of PCR mixture containing each nested primer pair, nehexAA1893-nehexAA1905 at 0.16 µM for adenovirus detection and 0.20 µM nEnt1-nEnt2 for enterovirus detection, in a new 30-cycle amplification (Table 2).

RT and PCR mixtures without DNA were used as negative controls and placed between every two or five samples for the specificity or field sample analysis, respectively. Twelve microliters of the amplified DNA mixture was analyzed for amplification products by gel electrophoresis on a 2% NuSieve GTG-1% SeaKem ME agarose gel (FMC Bioproducts, Rockland, Maine) and stained with ethidium bromide. To prevent samples from spilling from one lane into another, the samples were located in the gel on alternate lanes or consecutively if the amplified DNAs were expected to be of different sizes.

RESULTS

Concentration of viral particles for PCR or RT-PCR analysis from environmental samples. The most consistent results

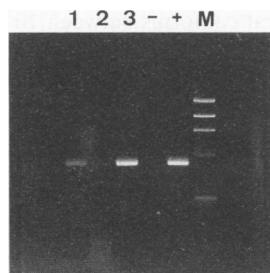


FIG. 1. Agarose gel electrophoresis showing amplified products of 534 bases in 30 cycles of amplification by using external enterovirus primers. Lane 1, RNA directly extracted from sewage (3.4 pg [estimated] of poliovirus type 1 RNA); lane 2, total nucleic acid extracted from the pellet of the same sewage sample concentrated by centrifugation; lane 3, RNA obtained by the method described in the text (70 pg [estimated] of poliovirus type 1 RNA); lanes - and +, negative and positive controls (100 pg [estimated] of poliovirus type 1 RNA), respectively; lane M, molecular weight standard marker ϕ X174 *Hae*III digest.

were given by treatment 4, which involved forming pellets of all the suspended solids with viral particles and eluting the viruses with glycine buffer. Total nucleic acid extraction directly from the pellet yielded negative results or only weakly positive results, and the use of low-protein-binding filters was rejected because of the high cost and poor results with some highly polluted samples. The method proposed is very easy and can be applied to samples with low and high levels of fecal contamination and has the additional advantage of avoiding inhibition of the reverse transcription and PCR amplification (Fig. 1), which is a common problem in many environmental samples (26). Results indicated that total nucleic acid extracted directly from the sample often needs further dilutions in order to give a positive amplification signal (data not shown). Studies on the efficiency of the viral recovery by this method were performed in three separate experiments by adding a highly concentrated stock of poliovirus type 1 (10^7 PFU) to 100 ml of sewage, with an average recovery of 60% of the poliovirus PFU previously added (50, 60, and 70% in the three experiments).

Nucleic acid extraction. The method described by Boom et al. (8), based on the adsorption of nucleic acids on silica particles, showed the highest recovery rate of the nucleic acids, between 118.9 and 13.04% more than that obtained by the method described by Chomczynski and Sacchi (10); the differences were higher in the samples with lower levels of viruses. These results were consistent with the more intense bands of amplified DNA observed in agarose gel electrophoresis, when the DNA extracted by this method was compared with that extracted by the method of Chomczynski and Sacchi (10). The glass powder method did not yield good recovery results in our assays and was abandoned.

Specificity of the primers for adenovirus and enterovirus detection. The adenovirus hexon primers used in the two-step amplification were shown to be able to detect the 47 human adenovirus serotypes described to date, all of them prototype or prototype-like strains (Table 1).

Both the external primers Ent1 and Ent2 and the nested primers neEnt1 and neEnt2 recognized the 24 different enterovirus strains assayed: echoviruses 2, 5, 6, 7, 9, 11, 15, 18, 19, 21, 25, 27, 29, and 30; coxsackieviruses A9, B1, B2, B3, B4, B5, and B6; and poliovirus types 1, 2, and 3.

Sensitivity of nucleic acid extraction, reverse transcription, and enzymatic amplification. Amplification of the superna-

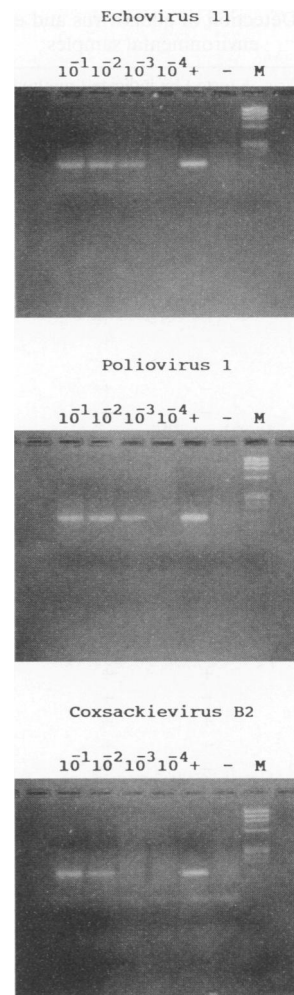


FIG. 2. Sensitivity of nested PCR for enterovirus detection. The gels show an agarose gel in which lane M contains a ϕ X174 *Hae*III digest, the molecular weight standard marker; lane + contains a positive control (100 pg [estimated] of poliovirus type 1 RNA); and lane - contains a negative control. PFU concentrations are indicated above the gels.

tants of the infected cells produced clearly visible bands in agarose gel electrophoresis up to the dilution corresponding to 10^{-3} PFU of the cDNA, corresponding to 1 PFU for poliovirus type 1 and echovirus 11, and to 10^{-2} PFU for coxsackievirus B2 (Fig. 2). Since it has been estimated that there is a particle infectivity ratio of between 100 and 1,000 for enteroviruses, we can calculate that the nucleic acid extraction, reverse transcription, and PCR amplification procedure used is able to detect the RNA corresponding to 1 to 10 enterovirus particles, at least from the data shown by the three enteroviruses checked.

The primers used for adenovirus detection have been shown in a previous study (3) to have a sensitivity as high as one purified viral particle when nested PCR amplification is applied.

Adenoviruses and enteroviruses in field samples. In this study, 16 sewage samples were analyzed for adenoviruses and enteroviruses; 12 (75%) were enterovirus positive in 2-ml PCR samples, and 16 (100%) were adenovirus positive in 4-ml PCR samples. All samples were taken during the months of April,

TABLE 3. Detection of adenovirus and enterovirus in environmental samples

Sample source and no.	Virus detected by indicated method ^a				PFU count of enterovirus ^b
	1-step PCR amplification		Nested PCR amplification		
	Adenovirus	Enterovirus	Adenovirus	Enterovirus	
River water					
1	+	—	+	+	0
2	+	—	+	+	0
3	+	—	+	+	0
4	+	—	+	+	0
5	+	—	+	+	0
6	—	—	+	+	0
7	—	—	+	+	0
8	+	—	+	+	2
9	—	—	+	+	0
Sewage					
1	+	+	+	+	0
2	+	+	+	+	2
3	+	+	+	+	0
4	—	+	+	+	0
5	—	+	+	+	0
6	+	+	+	+	0
7	+	—	+	—	0
8	—	+	+	+	0
9	+	—	+	—	0
10	+	+	+	+	12
11	—	—	+	—	3
12	—	—	+	—	0
13	—	—	+	+	0
14	—	—	+	+	2
15	—	—	+	+	3
16	—	—	+	+	0

^a +, detected; —, not detected.^b PFU per 20 ml of sewage sample and PFU per 2 liters of river water sample.

May, and July 1993. Only five sewage samples were also positive for enterovirus by PFU count in 10-ml cell cultures, with the values between 1 and 6 PFU per 10 ml of sample (Table 3). Only one sewage sample showed an enterovirus-positive result by PFU count (1.5 PFU per 10 ml) and a negative result by nested PCR amplification. All the sewage samples showed values of fecal coliforms between 10^5 and 10^6 CFU per 100 ml.

Of 20 sewage samples analyzed independently for adenoviruses by nested PCR and cell culture infection, plus one PCR amplification, only 5 (25%) were cell culture positive in the 20-ml-equivalent samples and 9 (45%) were nested PCR positive in 4-ml samples.

The viral concentrates of nine river water samples, all of which were taken in June 1993, were analyzed, and adenoviruses and enteroviruses were recovered from all of them by PCR. However, it was clear that in two-thirds of the samples (six samples), adenoviruses were already detected after the first 30 cycles of amplification, whereas in all river water samples enterovirus was detected in the agarose electrophoresis gel only after two PCR amplifications. All nine river water samples were positive for adenoviruses and enteroviruses by nested PCR in the 2-liter-equivalent river water sample, and only one was enterovirus positive, showing 2 PFU in 10 ml of the viral particle concentrate, which is equivalent to 5 liters of the river water sample. All nine of these samples were collected at hourly intervals, five on one day, and four on another day 1 week later. Samples from the Llobregat River regularly

show values of fecal coliforms between 10^3 and 10^4 CFU per 100 ml (28).

DISCUSSION

Nucleic acids can be extracted by the described method in 2 h without phenol or chloroform manipulation. The procedure for viral recovery and nucleic acid extraction from the environmental samples is relatively simple and can be applied indiscriminately to DNA and RNA viruses; specific pathogenic viruses such as hepatitis A virus can be detected by applying specific primer sets (data not shown).

A number of sewage and river water samples tested negative for enterovirus and adenovirus by the conventional cell culture approach for viral detection. Moreover, one-step PCR showed a number of samples that were false negative. Only the nested PCR showed a higher level of sensitivity of detection of these viruses. One of the most serious problems related to the use of the nested PCR technique is how to avoid the false positives easily obtained by contamination with amplified DNA (14). It is necessary to follow extremely carefully the generally recommended precautions by using disposable material, separate areas and materials for amplified and nonamplified samples, and tips with a membrane to avoid aerosol contamination, etc. We attempted to reduce the probability of amplifying contaminant DNA by treatment with *AluI* and uracil DNA glycosylase (13, 19). Those treatments did not interfere with the amplification and showed satisfactory results.

The primers designed for enterovirus detection show high levels of sensitivity and, in our opinion, specificity, which makes the detection by nested PCR amplification of the 138-bp region sufficient for the monitoring of enteroviruses in environmental samples.

Results of viral recovery and concentration procedures indicate that the treatment applied provides a method for concentration of the viral nucleic acids of different viruses from environmental samples, with a high applicability to samples with very different levels of fecal contamination, while removing or inactivating the inhibitors for the PCR and RT-PCR detected in some of the samples. This procedure is less complex and less costly for routine application than other methods previously described (1).

The results obtained in the sensitivity experiments for enterovirus detection are in accord with those obtained with the field samples, of which a large number were positive by PCR but negative by PFU count. Moreover, several samples showing 1 PFU per 20 ml were positive after 30 cycles of amplification in which the volume analyzed was the equivalent of a 2-ml sample. One sewage sample was enterovirus positive by PFU count but negative by nested PCR. This may be explained either by the presence of an enterovirus that was not detected by PCR or, according to other authors (18), by the presence of reoviruses, which are also able to grow on BGM cells and are sometimes present in environmental samples in greater numbers than enteroviruses.

It should be borne in mind that not all viral genomes detected correspond to infectious viral particles and a high proportion of noninfectious viral particles may be expected in the environment.

The method that we propose for the detection of adenoviruses and enteroviruses in environmental samples makes it possible to obtain information about the presence of viral contaminants in a few hours. This method provides for the detection of ≤ 10 particles of human adenovirus or enterovirus, which is 100 to 1,000 times higher than the sensitivity of cell

culture, gives new comparative data about the presence of adenoviruses and enteroviruses in wastewater, and shows a high prevalence of adenoviruses in sewage and in river water samples. These data suggest that the use of adenovirus positivity in a PCR sample as an indicator of viral pollution merits further attention. Further studies are also required in order to determine the extent and diversity of adenovirus contamination in sewage and polluted water over longer periods.

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